Pace, N. C. (1975), *Crit. Rev. Biochem. 3*, 1.
Perlmann, G. E. (1963), *J. Mol. Biol. 6*, 452.
Perlmann, G. E., Oplatka, A., and Katchalsky, A. (1967), *J. Biol. Chem. 242*, 5163.
Scatchard, G., Hamer, W. J., and Wood, S. E. (1938), *J. Am. Chem. Soc. 60*, 3061.
Schrager, R. J. (1970), *J. Assoc. Comput. Mach. 17*, 446.
Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.
Sturtevant, J. M. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236.
Tanford, C. (1968), *Adv. Protein Chem. 23*, 121.
Tanford, C. (1970), *Adv. Protein Chem. 24*, 1.
Tsong, T. Y., Baldwin, R. L., and McPhie, P. (1972), *J. Mol. Biol. 63*, 453.
White, F. H., Jr. (1976), *Biochemistry 15*, 2906.

Hydrolytically Induced Allosteric Change in the Heavy Chain of Intact Myosin Involving Nonessential Thiol Groups†

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ABSTRACT: The two globular head portions, each bearing an active site, contain an uncleaved heavy chain when isolated by chymotrypsin from intact myosin. By specific labeling with radioactive *N*-ethylmaleimide the essential thiol 1 and thiol 2 groups were found to reside in this heavy chain. In intact myosin nonessential thiol 3 groups become the most reactive during ATP hydrolysis above 15 °C. These thiol 3 groups are located in a portion of the myosin heavy chain which appears as a fragment with an apparent molecular weight of 11 000 during proteolysis. The facts that this fragment is produced in an almost 1:1 molar ratio with the head heavy chain and that it bears unblocked N-terminal amino groups whereas the heavy chain does not and is not contained in the rod portion of the myosin molecule indicate that it may originate from the heavy chains in the neck region where the heads are joined to the rod. Since this fragment is removed by ion-exchange chromatography, it is not part of the functioning head and hence not involved in the active site. As its nonessential thiol 3 groups are rendered the most reactive of all thiol groups in the enzyme–product complex M**ADP·P_i, the hydrolytic step induces an allosteric conformational change in the neck region of intact myosin.

Monitoring the changes in reactivity of specific thiol groups has acquired widespread recognition as a method for probing protein conformational changes. These side groups may become even more reactive than they are in the free amino acid cysteine as a result of local environmental changes, which in turn indicate alterations in chain conformation (Gutfreund and McMurray, 1970). Enzymes are known to undergo conformational changes following the binding of substrate and the subsequent steps of their catalytic activity. These latter syncatalytic alterations in the protein structure can affect the environment of reactive side groups of specific amino acids as has been demonstrated for a certain cysteinyl residue of aspartate aminotransferase whose activity is markedly enhanced during catalysis (Birchmeier et al., 1973).

The hydrolysis of Mg–ATP by isolated myosin heads, the soluble proteolytic fragment of myosin bearing an active site, has been shown by fast kinetic techniques to proceed via seven elementary steps (Bagshaw and Trentham, 1974). The long lived species within this hydrolytic cycle is known to be an enzyme–product complex M**ADP·P_i[1] (Lymn and Taylor, 1970; Trentham et al., 1972). It can be distinguished from the complex occurring on direct binding of the product (M*ADP) which has also been shown to represent a reaction cycle intermediate (Bagshaw and Trentham, 1974), on the grounds of intrinsic tryptophan fluorescence (Werber et al., 1972; Mandelkow and Mandelkow, 1973), ultraviolet absorption (Morita, 1967; Malik and Martonosi, 1972), electron spin-label techinques (Seidel and Gergely, 1971), and circular dichroism (Murphy, 1974). A further approach is that of thiol group reactivity (Watterson and Schaub, 1973; Reisler et al., 1974) which does not involve spectral techniques and so may be done on insoluble myosin suspensions. It allows characterization of the different forms of the enzyme with or without ligand independent of a reference state, say the enzyme alone. This is achieved by following the sequence of blockage of different types of thiol groups, under a given condition at high or low ionic strength, in conjunction with the effect of this progressive blockage on the enzymic properties of myosin (Watterson et al., 1975).

Several types of thiol groups have been classified on the basis of the effect of their blockage on the myosin ATPase. Blockage of thiol 1 causes activation of the Ca^{2+}-dependent ATPase, when tested at high ionic strength, and concomitant inactivation of the K^+-dependent ATPase, while subsequent blockage of thiol 2 then inactivates the Ca^{2+}-dependent ATPase (Sekine et al., 1962; Sekine and Yamaguchi, 1963). Blockage of thiol 2 alone seems only to inactivate the K^+-dependent ATPase without appreciably affecting the Ca^{2+}-dependent ATPase (Reisler et al., 1974). These groups will be referred to as the essential thiol groups and they have recently been shown to

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[1] Abbreviations used: ATPase, adenosine-5′-triphosphatase (EC 3.6.1.3); HMM, heavy meromyosin; HC, heavy chain; LC-1, light chain 1; LC-2, light chain 2; LC-3, light chain 3; P_i, inorganic phosphate; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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reside in the HC of myosin from fast, slow, and heart muscle (Pfister et al., 1975). A third category, the so-called thiol 3 groups, has further been described (Schaub et al., 1975). These groups are referred to as nonessential since their blockage, in intact myosin, has no effect on the enzymic properties.

In this work the subunit distribution of incorporated activity after labeling of specific thiol groups, in the presence of either substrate or product, was examined on myosin, HMM, and isolated myosin heads. Furthermore, the incorporated radioactivity was followed in the various proteolytic fragments and their subunits after labeling intact myosin in order to establish their positions in the molecule. The results lead to the speculation that the relevant nonessential thiol 3 groups are located in a part of the myosin whose structural changes are caused allosterically by the step occurring during hydrolysis. This part seems to be located somewhere between the globular heads and the rod-like HMM-subfragment 2 in the neck region of the molecule. Although this region and its movements are as yet very undefined, its flexibility has been described as the cause of spectroscopic signals from labels attached to thiol groups in myosin (Mendelson et al., 1973; Thomas et al., 1975). Furthermore, the model of cross-bridge movement proposed by Huxley and Simmons (1971) requires the head portions to rotate relative to the rod portion; i.e., a flexible hinge region is visualized somewhere in the connecting neck area. After detachment the movement in the opposite direction has been postulated to be closely coordinated with the actual hydrolysis of Mg-ATP on the cross-bridge (Mannherz et al., 1973).

Materials and Methods

Distilled and deionized water was used throughout. Inorganic salts were of analytical grade and other reagents of the highest purity obtainable. *N*-Ethyl[1-^{14}C]maleimide and [1-^{14}C]iodoacetamide were obtained from New England Nuclear Co., Boston, Mass.; α-chymotrypsin of bovine pancreas three times crystallized and soybean inhibitor type 1-S were from Sigma Chem. Co., St. Louis, Mo.; trypsin-L-1-tosylamido-2-phenylethyl chloromethyl ketone was from Worthington Biochem. Corp.; and crystalline papain was from Boehringer Mannheim GmbH, Mannheim, West Germany.

Myosin and its fragments were prepared from fast skeletal rabbit muscles as described elsewhere: myosin (Schaub et al., 1975); HMM and HMM-subfragment 1, i.e., myosin heads, with chymotrypsin (Weeds and Taylor, 1975); heads with papain (Margossian et al., 1975); heads with trypsin (Biro et al., 1973); entire myosin rod, i.e., light meromyosin plus HMM-subfragment 2 (Weeds and Pope, 1977). Chromatography of head preparations was performed on DEAE-Sephadex A-50 according to Weeds and Taylor (1975). The HC of myosin and of heads prepared by chymotrypsin were separated from the light chains by chromatography on Sephadex G-100 in 6 M guanidine hydrochloride and 20 mM Tris-Cl buffer, pH 8.5 (column dimensions: 2 × 150 cm) after carboxymethylation in the column solvent with radioactive iodoacetamide to help monitor the protein eluate (Schaub and Perry, 1971).

Alkylation with radioactively labeled NEM of myosin, HMM, and heads was carried out with a 2–100 M excess of reagent over protein in 25–50 mM Tris-Cl buffer, pH 7.6 or 8.0, for 2–30 min under different conditions indicated in the text as described in details elsewhere (Watterson et al., 1975). Incorporation of radioactivity was assessed by liquid scintillation techniques and the K^+- as well as the Ca^{2+}-dependent ATPase activities were measured. For calculations the following molecular weights were taken: myosin, 470 000 (Dreizen et al., 1967); HMM, 340 000 (Mueller, 1964); myosin head, 120 000 (Weeds and Taylor, 1975); and myosin rod, 220 000 (Lowey et al., 1969).

Electrophoresis in $NaDodSO_4$ of myosin on 7.5% and heads or HMM on 7.5 and 10% polyacrylamide gels (column dimensions: 5 × 80 mm) was carried out according to Dunker and Rueckert (1969) in 20 mM phosphate buffer, pH 7.0, or in 82.5 mM Tris/400 mM boric acid, pH 7.5 (Cummins and Perry, 1973), using the fixation, staining, and destaining procedure with Coomassie brilliant blue R-250 of Sobieszek and Bremel (1975). Destained gels were scanned on a densitometer, Bender and Hobein Model Integraph CH. Apparent molecular weights were determined using myosin HC, phosphorylase *a*, bovine serum albumin, actin, pepsin, trypsin, ribonuclease, and cytochrome *c* as marker proteins. For determination of subunit stoichiometry it was assumed that all protein bands originating from myosin and its fragments stained with equal specific intensity using tropomyosin as reference protein. For these determinations the chemical molecular weights of 20 700 for LC-1, 19 000 for LC-2, and 16 500 for LC-3 (Weeds and Lowey, 1971; Frank and Weeds, 1974) were used. For assessment of radioactivity incorporated into the electrophoretically resolved subunits, the gels were sliced into 2-mm sections either after the standard staining procedure or immediately after electrophoresis without fixation or staining. The gel slices were dissolved in 0.3 mL of 30% hydrogen peroxide for 40 h at 50 °C (Gray and Steffensen, 1968) and counted for radioactivity in a liquid scintillation spectrometer with an approximate efficiency of 60%. No additional quenching arising from the dissolved gel was observed.

The total number of thiol groups in heads prepared by chymotrypsin was determined after alkylation of the denatured protein with radioactive NEM or iodoacetamide as described by Joernvall (1970). A modified Edman procedure (Iwanaga et al., 1969) was used for analysis of N-terminal amino acids. The phenylthiohydantoin-amino acid derivatives were identified directly on thin-layer chromatography (Kulbe, 1974) and after regeneration by hydrolysis in 6 M HCl containing 0.1% $SnCl_2$ for 4 h at 150 °C (Mendez and Lai, 1975) on a Labotron model Liquimat-2 amino acid analyzer by standard procedures.

Ca^{2+}- and K^+-dependent ATPase activities were measured (Fiske and SubbaRow, 1925) at 25 °C, pH 7.6, and high ionic strength as specified earlier (Schaub et al., 1975). All buffer solutions were prepared at the temperature of use. Protein concentrations were determined by the biuret reaction or according to Lowry et al. (1951) standardized by ultramicro-Kjeldahl estimation of nitrogen (Strauch, 1965).

Results

Incorporation of Radioactive [^{14}C]NEM into Myosin, HMM and Isolated Myosin Heads. It has been earlier reported that only about 4 of the total of 42 thiol groups in myosin (Kominz et al., 1954; Barany et al., 1964; Huszar and Elzinga, 1971) react readily with [^{14}C]NEM at 25 °C, pH 7.6, and low ionic strength in the presence of Mg-nucleotides (Schaub et al., 1975). In the presence of Mg-ADP these 4 were found to comprise the 4 essential thiol groups since the Ca^{2+}-dependent ATPase became fully inactivated. When the alkylation was carried out in the presence of Mg-ATP, i.e., during hydrolysis when the enzyme-product complex M**ADP-P_i (Bagshaw and Trentham, 1974) is the predominant intermediate, nonessential thiol groups whose blockage does not affect the enzymic properties do also react readily with

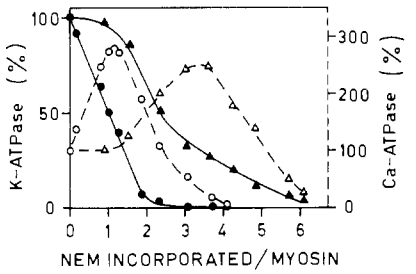

FIGURE 1: Effect of incorporation of [^{14}C]NEM into myosin on relative ATPase activities. Prior to ATPase tests alkylation was performed on 5.4 μM myosin in the presence of 7.5 mM $MgCl_2$, 30 mM KCl, and 5 mM ADP (● and ○) for 5 min or 50 μM ATP (▲ and △) for 2 min at 25 °C and pH 7.6. Full symbols: K^+-dependent ATPase. Open symbols: Ca^{2+}-dependent ATPase.

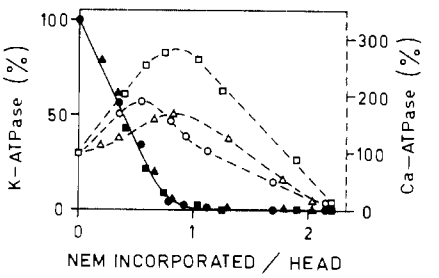

FIGURE 2: Effect of incorporation of [^{14}C]NEM into myosin heads prepared by chymotrypsin on relative ATPase activities. Prior to ATPase tests alkylation was performed on 7.5 μM heads in the presence of 10 mM $MgCl_2$, 20 mM KCl, and 5 mM ADP (● and ○) or ATP (▲ and △) for 10 min at 25 °C and pH 8.0, and on 3.6 μM heads in the presence of 10 mM $MgCl_2$ and 20 mM KCl (■ and □) for 15 min at 0 °C. Full symbols: K^+-dependent ATPase. Open symbols: Ca^{2+}-dependent ATPase.

NEM. The initial lag phase evident in both K^+- and Ca^{2+}-dependent ATPases indicates that a thiol 3 group is in fact the most reactive during turnover of Mg–ATP at 25 °C and another 1–3 nonessential groups reacted along with the essential ones, so that 6–8 groups had to be blocked in order to fully inactivate the enzyme (Figure 1). The same difference in incorporation pattern was observed when the alkylation was performed at high (in the millimolar range) as well as at low concentrations of substrate or product. In the presence of Mg–ATP in a molar excess of only 10–20 times over myosin (i.e., 5–10 times molar excess over active sites) the alkylation reaction had to be terminated after 1–3 min to ensure that most of the active sites were still in their long lived intermediate state. The results were identical with those with nucleotide concentrations ranging from 30 μM to 5 mM. This observation makes it rather unlikely that the increased reactivity of nonessential thiol groups during hydrolysis above 15 °C is due to some additional "nonspecific" binding of ATP to sites other than the active ones at high substrate concentrations. The experiments with Mg–ADP and Mg–ATP were also performed in the additional presence of either 10^{-4} M $CaCl_2$ or 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) without changing the results. Similar differences between the types of thiol groups rendered most reactive by Mg–ADP or Mg–ATP could be demonstrated with radioactively labeled iodoacetamide but not with iodoacetic acid, fluorodinitrobenzene, *p*-chloromercuribenzoic acid or 5,5'-dithiobis(2-nitrobenzoic acid).

Parallel incorporation studies on HMM in the presence of either ADP or ATP at 25 °C yielded similar results to those obtained with myosin. The effects of alkylation on the ATPase activities indicate that the presence of substrate also produces a conformation in which a nonessential thiol group is the most reactive. At least one further nonessential group reacts along with the essential ones before the K^+-dependent ATPase is abolished, whereas in the case of Mg–ADP, as with myosin, the four essential thiol groups react first.

The same type of experiments was also performed on isolated heads prepared by tryptic digestion of myofibrils (Biro et al., 1973) or digestion of myosin with papain (Margossian et al., 1975) or chymotrypsin (Weeds and Taylor, 1975). About two thiol groups were blocked with NEM under all conditions. These two groups comprised almost exclusively the two essential thiol groups since the K^+- and Ca^{2+}-dependent ATPases were inactivated after incorporation of the first and second mole of NEM, respectively (Figure 2). This result leads directly to the conclusion that in all three types of head preparations each head contained just one of each essential thiol group, under the assumption that each head contains an active site. However, the sequence of blockage of these two groups was to some degree influenced by the presence of a ligand during the alkylation. This is reflected in the position and the degree of maximum activation seen in the Ca^{2+}-dependent ATPase curves in Figure 2. In the absence of nucleotides thiol 1 reacts clearly more readily than thiol 2, whereas in the presence of the nucleotides both types of essential thiol groups appear to react concomitantly. Since in isolated heads only the essential thiol 1 and thiol 2 groups react readily with NEM, it is no surprise that the incorporation pattern does not distinguish between the species M*ADP and M**ADP·P_i. It was found, however, that the rate of reaction of the essential groups differs significantly between the two cases. In the presence of Mg–ADP the rate of incorporation of NEM is about twice that with Mg–ATP. The total number of thiol groups per head prepared with chymotrypsin was estimated on six preparations yielding 10.3 ± 0.8 per head. Even higher values of 12–13 thiol groups per 120 000 daltons have been reported (Mueller, 1965; Jones and Perry, 1966; Lowey et al., 1969; Huszar and Elzinga, 1971). In all these cases, however, the heads were prepared in the absence of EDTA leading to a variable degree of LC-2 retained in them (Kendrick-Jones, 1974; Margossian et al., 1975). As this light chain contains two thiol groups (Weeds and Lowey, 1971; Collins, 1976), its presence could explain the higher values compared to that given here. This means that there are at least about eight thiol groups in addition to the two well defined essential ones per head, none of which exhibits reactivity toward NEM in the experiments described above. In particular, there is no syncatalytic exposure of thiol 3 groups during turnover of Mg–ATP. It is thus of interest to discover whether the thiol 3 groups which can be exposed in myosin are present in the isolated head subfragment.

Subunit Identification in NaDodSO$_4$ Electrophoresis. Identification of protein subunits of myosin and its proteolytic fragments has relied on evidence from the electrophoretic mobility in the presence of $NaDodSO_4$. Figure 3A shows superimposed densitometric tracings of myosin and heads prepared by chymotrypsin which had not been subjected to ionic exchange chromatography. The head pattern invariably showed complete loss of LC-2 but an additional band arising from a degraded fragment migrating faster than LC-3. Its electrophoretic mobility was markedly lower in the Tris–borate buffer system so that it overlapped with the band of LC-3. This was never the case in the phosphate buffer system and hence the latter was used for all electrophoretic results reported here. No difference in migration of all three types of light chains was observed in the two buffer systems. Since prior to digestion with chymotrypsin the parent myosin was alkylated in the presence

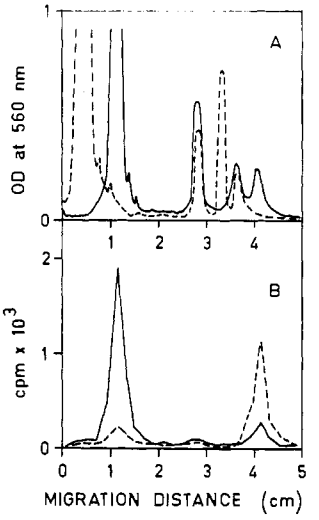

FIGURE 3: Distribution of radioactivity over subunits of heads prepared with chymotrypsin from myosins which had been alkylated with [^{14}C]-NEM in the presence of either Mg-ADP or Mg-ATP. Sodium dodecyl sulfate electrophoresis was performed on 7.5% polyacrylamide gels in the phosphate buffer system. Tracings of different gels of the same run are superimposed. (A) Electrophoretograms (OD values given in arbitrary units) of myosin (36 μg, broken line) containing 1.60 mol of [^{14}C]NEM incorporated in the presence of Mg-ADP and its corresponding head preparation (21 μg, full line). The peak maxima with increasing migration distance represent myosin HC, head HC, LC-1, LC-2, LC-3, and the 11 000 daltons fragment. (B) Radioactivity recovered from gels of heads prepared from myosins containing 1.60 and 1.19 mol of [^{14}C]NEM incorporated in the presence of Mg-ADP (full line) and Mg-ATP (broken line), respectively.

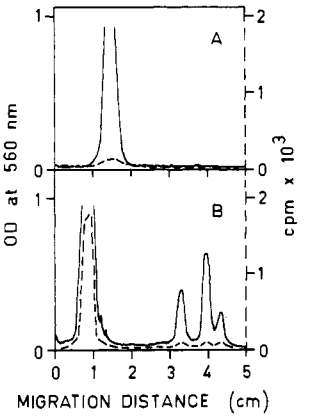

FIGURE 4: Distribution of radioactivity over myosin rod and HMM subunits prepared by chymotrypsin. NaDodSO$_4$ electrophoresis was performed on 7.5% polyacrylamide gels in the phosphate buffer system. OD values of tracings are given in arbitrary units (full line). Recovered radioactivity (broken line). (A) Loaded on gel were 25 μg of rod prepared from myosin containing 0.85 mol of [^{14}C]NEM (i.e., about 47 000 cpm per mg of myosin) incorporated in the presence of Mg-ATP at 25 °C and pH 7.6. (B) Loaded was 31 μg of HMM containing 1.15 mol of [^{14}C]NEM incorporated in the presence of Mg-ATP at 25 °C and pH 7.6. The peak maxima with increasing migration distance represent HC of HMM, LC-1, LC-2, and LC-3.

of either nucleotide or without ligand, it had to be examined whether the originating head preparations were identical. The band patterns in NaDodSO$_4$ electrophoresis from head preparations from differently modified myosin samples done in parallel, looked identical. On mixing them the corresponding protein bands comigrated. Furthermore, the relative areas of the protein bands in the low molecular weight region originating from such differently modified parent myosins proved to remain constant in one experiment.

Apparent molecular weights of 90 000 ± 4600, 24 800 ± 900, 17 500 ± 1100, 14 700 ± 500, and 10 800 ± 900 corresponding to head HC, LC-1, LC-2, LC-3, and the degraded fragment, respectively, were calculated from gels of 12 myosin and 31 head preparations. The protein bands corresponding to HC of HMM and to the entire myosin rod after the heads had been cleaved off were, in three preparations, observed to migrate in the molecular weight range of 120 000–140 000 and 100 000–120 000 daltons, respectively. In particular the HMM preparations did not display protein bands corresponding to the 11 000 daltons fragment nor to the 90 000 head HC (Figure 4).

The 11 000-daltons fragment could be separated from the heads by chromatography on DEAE-Sephadex. It was not adsorbed on the resin and its removal did not affect the enzymic properties nor alkylation results of the heads, indicating that it is not an integral part of the head subfragment. NaDodSO$_4$ electrophoresis of the purified degraded fragment sometimes revealed slight heterogeneity consisting of accompanying minor protein bands on both sides of the 11 000-daltons position. The head fraction itself resolved into two overlapping peaks bringing about partial separation with respect to the LC-1 and LC-3 subunits when desorbed from DEAE-Sephadex by the NaCl gradient. The band patterns in NaDodSO$_4$ gel electrophoresis before and after chromatography resembled closely those published by Weeds and Taylor (1975).

Digestion of insoluble myosin in the presence of EDTA was carried out for different periods of time and the solubilized protein fraction examined in NaDodSO$_4$ electrophoresis. The results revealed that within 1.5 min a band migrating just ahead of the LC-3 appeared which probably represented the 14 000-daltons fragment of LC-2 reported by Weeds and Pope (1977). Under our digestion conditions this band faded away already after 3 min and disappeared completely at 5 min. The 11 000-daltons fragment, on the other hand, migrating faster than the latter band began to appear later and increased in amount in proportion to the 90 000-daltons head HC. Both the 90 000- and 11 000-daltons bands remained almost constant in proportion over 20 min of digestion time. In a number of head preparations the molar ratio of LC-1 and LC-3 to HC was found to be on the average 0.65 and 0.45, respectively. This means that there is just one of either light chain associated with one head. Thence the heads containing an LC-1 predominate over those with an LC-3. This uneven distribution reflects that of LC-1 and LC-3 found in the parent myosin preparations and corresponds to values reported in the literature for myosin from fast contracting skeletal muscles (Sarkar, 1972; Weeds et al., 1975). To estimate the molar amount of the 11 000-daltons fragment, its staining intensity was compared with those of the LC-1 and LC-3 bands in gels for 22 preparations of crude heads before chromatography. With the proviso that there is just one light chain, i.e., either a LC-1 or LC-3, per head an average molar ratio of 0.73 ± 0.24 was calculated for the degraded fragment based on a molecular weight of 10 800. This would indicate that there is about one such fragment per isolated head in these preparations.

Subunit Distribution of Incorporated Radioactive [^{14}C]-*NEM.* In the majority of experiments for localization of specifically labeled thiol groups in different parts of the myosin, heads were prepared by chymotrypsin since their HC proved to remain uncleaved. After incorporation of up to 6 mol of NEM per myosin, the recovery of radioactivity in the crude head preparations in 29 cases was on average 108 ± 15% on a molar basis. This indicates that during alkylation in the presence of a nucleotide all NEM was incorporated into the head portion of myosin exclusively.

TABLE I: Incorporation of [^{14}C]NEM under Various Conditions at pH 7.6 into Myosin HC and Its Distribution over the Head HC and 11 000-Daltons Fragment of the Corresponding Head Preparations Assessed from NaDodSO$_4$ Gel Electrophoresis as Described in the Text.

Alkylation conditions for parent myosins		Incorp of [^{14}C]NEM into myosin		Distribution of [^{14}C]NEM over head HC and 11 000-daltons fragment	
Nucleotide	Temp (°C)	Mol per myosin	% per both HC	% per head HC	% per fragment
ADP (6)[a]	0	0.9–5.8	91 ± 5	77 ± 6	24 ± 9
ADP (6)[a]	25	0.6–3.7	93 ±5	85 ± 7	16 ± 2
ATP (6)[a]	0	0.8–4.1	93 ± 4	88 ± 8	14 ± 5
ATP	25	0.60	95	41	65
		0.64	90	20	78
		0.87	91	40	59
		1.07	92	38	57
		1.19	90	33	66
		1.19	86	21	71
		1.25	88	62	45
		2.17	89	56	41
		2.60	85	71	30
		3.69	91	76	25
		5.03	89	69	31

[a] Number of experiments.

The recovery of radioactivity from gels after NaDodSO$_4$ electrophoresis which were sliced and counted after fixation and staining was between 60 and 90%. Within one electrophoretic run, however, all gels exhibited the same percentage of recovery. In 15 gels which were sliced immediately after electrophoresis without prior fixation or staining the recovery was 99 ± 10%.

In myosin the major portion of radioactivity incorporated in the presence of a nucleotide was invariably recovered in the HC band, so that in none of these cases was any specific labeling observed in the light chains (Table I). The radioactivity was distributed more or less evenly over the three types of light chains averaging 9% in all cases of the total incorporated per myosin. Together the 2 mol of LC-2 per myosin never contained more than 6%. Under no condition of alkylation was any appreciable amount of radioactivity recovered in the doublet or sometimes triplet set of very fine bands migrating just ahead of the myosin HC in the region where C- and M-protein bands are reported to occur (Offer, 1972).

A marked difference in the subunit distribution of radioactivity was found, however, in the crude heads prepared from such labeled myosins depending on the nucleotide present during alkylation. In the two cases shown in Figure 3B, between 1 and 2 mol of NEM were incorporated per myosin either in the presence of Mg–ADP or Mg–ATP. In the former case 97% and 86% in the latter were incorporated into the HC of the parent myosins. After digestion with chymotrypsin a reciprocal distribution of the radioactivity over the head HC and the degraded fragment was observed. While the majority of counts remained on the head HC in the case of Mg–ADP, the amount recovered on the degraded fragment was more than three times greater than that on the HC in the case of Mg–ATP.

The way in which the radioactivity originally present in the myosin HC was distributed over the HC and 11 000-daltons fragment of subsequently prepared heads was quantitatively evaluated with progressive degrees of incorporation of NEM in the presence of product or substrate. In all 29 cases contained in Table I the relative recovery of radioactivity in the 11 000-daltons fragment was 3–25 times more than that of the two LC-2 of the corresponding myosins. On the other hand, only the subunits LC-1 and LC-3 appear unaltered in the head preparations with chymotrypsin as judged from their electrophoretic behavior. As these latter subunits bear insignificant amounts of radioactivity, none of the light chains can account for the radioactivity found in the 11 000-daltons fragment. It thus seems that this fragment must be derived from the myosin HC. Then the amount of radioactivity incorporated in the myosin HC is expected to become divided between the 90 000- and 11 000-daltons polypeptide chains in the head preparations. The relative amounts of radioactivity found in these two fragments in the gels of heads were calculated after correction eliminating the small contribution of LC-1 and LC-3 carried over from the parent myosins. Due to this correction the sum of the relative amounts of radioactivity is not exactly 100% (Table I).

In all cases when alkylation was performed in the presence of Mg–ADP at 25 or 0 °C, around 90% of the radioactivity was in the myosin HC and of this around 80% remained in the HC of heads whatever the degree of incorporation of NEM. However, with Mg–ATP at 25 °C, where again around 90% of the radioactivity was located in the myosin HC, the relative amounts recovered in the head HC and degraded fragment varied inversely as the degree of incorporation of NEM into myosin increased. With up to about 1 mol of NEM per myosin only around 30% of the radioactivity appeared in the head HC, whereas the remainder occurred in the 11 000-daltons fragment. Since the high proportion of radioactivity in the degraded fragment declined as the overall degree of incorporation into myosin increased, a larger number of thiol groups located on that part of the myosin HC which constitutes the head HC becomes blocked during turnover of Mg–ATP. On the other hand, after alkylation in the presence of Mg–ATP at 0 °C no appreciable amount of radioactivity was recovered in the 11 000-daltons fragment and its subunit distribution was identical with that found in the case of Mg–ADP. This latter result is also supported by those from alkylation studies performed in the presence of Mg–ATP as a function of temperature (Watterson et al., 1975). It was found that the difference in incorporation patterns between the cases of Mg–ADP and Mg–ATP seen at high temperature gradually disappeared, and they became identical at temperatures below 5 °C.

Although the recovered radioactivity in the head fraction accounted for the total incorporated in the parent myosins, the residual rod portions were also examined. After cleavage of the heads this part of the molecule consists of the tail portion

TABLE II: Incorporation of [^{14}C]NEM under Different Conditions at 25 °C and pH 8.0 into Chymotryptic Myosin Head Preparations Containing the 11 000-Daltons Fragment and Its Subunit Distribution Assessed from $NaDodSO_4$ Gel Electrophoresis.

Nucleotide present during alkylation	Mol of [^{14}C]NEM incorp per 120 000 daltons	Distribution of incorp radioact. over subunits (%)			
		HC	LC-1	LC-3	11 000-daltons fragment
None	1.90	71.5	11.3	7.8	9.4
	1.98	66.3	14.7	9.3	9.7
ADP	2.46	72.6	13.2	7.7	6.5
	2.24	75.0	11.5	7.3	6.2
ATP	1.53	63.7	16.8	9.4	10.1
	2.36	59.9	18.4	12.3	9.4

(light meromyosin) and HMM–subfragment 2. In three experiments with an incorporation of less than 1.2 mol of NEM per myosin in the presence of Mg–ATP at 25 °C, where the majority of radioactivity was in the 11 000-daltons fragment, the entire myosin rod was isolated and electrophoretically identified (Figure 4A). Two moles of rod was found to contain together only 5–10% of NEM originally present in the parent myosins.

A quite different picture emerges from the reaction of NEM directly with crude head preparations containing the 11 000-daltons fragment produced by chymotrypsin (Table II). In no case was there a specific labeling of the degraded fragment even when a 50 times molar excess of the reagent over the protein was used. Such extreme conditions are necessary in order to incorporate more than 2 mol of NEM per head since, as reported above, the nonessential thiol groups are well protected against blockage. Although relatively more counts were recovered on LC-1 and LC-3 than when the incorporation was done on intact myosin, the bulk of the radioactivity was located on the head HC, in particular, it made no difference whether the alkylation was performed in the presence of substrate or product at 25 °C.

With HMM preparations, which do not contain the 11 000-daltons fragment, the subunit distribution also revealed no difference between ADP and ATP. In both cases over 80% of the radioactivity was invariably recovered in the HC with degrees of incorporation of up to 3.6 mol of NEM per HMM (Figure 4B).

N-Terminal Amino Acid Analyses of the 11 000-Daltons Fragment. It is known that the N-terminal amino groups of both myosin HC are in the head region and that they are acetylated (Starr and Offer, 1973). In addition, the N-terminal amino groups of all three types of light chains have also been shown to be blocked (Frank and Weeds, 1974; Collins, 1976). Since such blocked amino groups do not react with phenyl isothiocyanate, head HC, the degraded fragment, the whole set of light chains prepared from intact myosin and crude head preparations after dialysis were separately subjected to the Edman degradation procedure. After purification on DEAE-Sephadex the head HC were separated from LC-1 and LC-3 by chromatography on Sephadex G-100 in 6 M guanidine hydrochloride. The set of light chains was separated from the myosin HC by the same chromatographic system prior to the Edman degradation. Phenylthiohydantoin-amino acid derivatives were liberated only from the purified 11 000-daltons fragment and crude head preparations. They were identified as Ala, Asp, and in lesser amounts Leu, Lys, and Gly. Identical results were obtained from three different preparations of the 11 000-daltons fragment. In contrast, the Edman procedure yielded no phenylthiohydantoin-amino acid derivatives from head HC or myosin light chains.

Discussion

It has been reported earlier that enzymically active isolated myosin heads contain the essential thiol 1 group (Seidel et al., 1971; Duke et al., 1976). In addition the results presented here show that isolated heads prepared by different techniques contain just one of each thiol 1 and thiol 2 groups, and that these are the only out of a total of at least ten groups which react readily with NEM in the presence or absence of nucleotides. This is the more surprising when one considers that in intact myosin as well as in HMM in the presence of Mg–ATP at 25 °C where the product complex M**ADP·P_i (Lymn and Taylor, 1970; Trentham et al., 1972) prevails, nonessential thiol 3 groups are rendered the most reactive. Although all three classes of thiols are in the myosin HC, only thiol 1 and thiol 2 are to be found in the 90 000-daltons HC of chymotryptic heads. On the other hand, the 11 000-daltons fragment whose removal does not impair the active site contains thiol 3. Since this fragment is not an integral part of the functioning head, one may conclude that the hydrolytic step causes allosteric changes in regions of the myosin molecule not involved in forming the active site.

The results of alkylation performed at low ATP concentrations seem to rule out the possibility that the conformation of the species M**ADP·P_i observed in intact myosin is due to additional nonspecific binding of excess substrate to sites other than the active ones. Further evidence for this is provided by the incorporation pattern obtained from alkylation in the presence of high concentrations of the ATP analogues adenosine 5′-(β,γ-imido)triphosphate and adenosine 5′-*O*-(3-thiotriphosphate). In both cases the enzyme–substrate complex is the predominant species (Yount et al., 1971; Bagshaw et al., 1972) and no thiol 3 groups become blocked as opposed to the case with ATP (Watterson et al., 1975). Results from direct binding studies with ATP analogues overwhelmingly indicate that myosin contains only two sites binding them (Weber and Murray, 1973). To our knowledge only the purine disulfide analogue 6,6′-dithiobis(inosine 5′-(β,γ-imido)triphosphate) has been shown to bind to myosin as well as to isolated heads at other sites where it gets covalently attached to cysteinyl residues (Wagner and Yount, 1975a,b). It has also been shown on myosin that the incorporation pattern with [^{14}C]NEM at high ATP concentrations approaches that obtained in the presence of ADP on lowering the temperature during the alkylation reaction. Below 5 °C they were found to be the same, i.e., thiol 3 groups no longer become blocked. This was interpreted as indicating a shift in the rate-limiting step in the ATP hydrolytic cycle so that on lowering the temperature the species M*ADP gradually becomes more abundant at the expense of M**ADP·P_i (Watterson et al., 1975). Kinetic studies also indicate that at low temperature the enzyme–product complex

M*ADP is the predominant species during ATP hydrolysis (Taylor et al., 1970; Malik and Martonosi, 1972; Bagshaw and Trentham, 1974). These arguments strongly confirm the deduction that hydrolysis above 15 °C is responsible for the observed allosteric conformational change and that exposure of nonessential thiol 3 groups is not merely caused by binding of substrate either at the active or other sites.

These thiol 3 groups have unequivocally been located on the myosin HC. Since they are, however, not contained in the entire rod portion comprising the myosin tail (light meromyosin) plus the adjoining HMM-subfragment 2, they seem to reside further along in the HC closer toward the N terminus whose acetylated end groups are in the myosin heads (Starr and Offer, 1973). This is indeed the case as they are contained in HMM-HC which in turn represent the N-terminal side of the myosin HC including HMM-subfragment 2 again. On the other hand, the 11 000-daltons fragment which contains the thiol 3 groups occurs in the crude head preparations in a near stoichiometric ratio of 1:1 to the 90 000-daltons head HC. Though it is not part of the fully functioning isolated head, it does seem to be connected somehow with the head region of the myosin molecule. Since the band pattern in $NaDodSO_4$ gels gave only one sharp band in the 90 000-daltons range, it can be concluded that the fragment must originate from one end rather than from somewhere in the middle of the head HC. In the latter case one would expect a number of bands with molecular weights smaller than 90 000 daltons. The results from end group analyses indicate that the head HC still bear their blocked N-terminal amino groups. This is in agreement with the results of Pope et al. (1977) who isolated the N-terminal peptides from the HC of such head preparations. Since only the degraded fragment contains unblocked N-terminal amino groups, it must have been cleaved off from the C-terminal end of the 90 000-daltons fragment. One may conclude, therefore, that the 11 000-daltons fragment originates from the neck region of the myosin HC where the heads are joined to the rod. Although the chymotrypsin seems to split the fragment out of a rather defined region of the myosin HC, one would not expect it to hydrolyze only one single peptide bond at both ends. Some heterogeneity was indeed observed in $NaDodSO_4$ electrophoresis of the purified fragment preparations which fits the fact that several free N-terminal amino acid residues were identified.

It has been reported that HMM contains around 25 cysteinyl residues (Lowey and Cohen, 1962; Jones and Perry, 1966; Lowey et al., 1969) per 340 000 daltons and differently prepared head preparations 12-13 residues (Mueller, 1965; Jones and Perry, 1966; Lowey et al., 1969; Huszar and Elzinga, 1971) per 120 000 daltons, i.e., 24-26 in the two heads of one myosin. On the other hand, Lowey et al. (1969) found 4 cysteinyl residues to reside in HMM-subfragment 2; i.e., 2 residues per chain, which, being part of the rod, joins the neck region from the end opposite to the heads. The balance of these numbers leaves hardly any cysteinyl residues to be located in the neck region where HMM-subfragment 2 and heads are joined together. This in turn ties in well with the finding that only 1-2 nonessential thiol 3 groups per HC become reactive in intact myosin during ATP hydrolysis. In fact amino acid analyses (unpublished results) indicate that the 11 000-daltons fragment contains just 2 cysteinyl residues.

References

Bagshaw, C. R., and Trentham, D. R. (1974), *Biochem. J. 141,* 331-349.

Bagshaw, C. R., Eccleston, J. F., Trentham, D. R., Yates, D. W., and Goody, R. S. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 127-135.

Barani, M., Gaetjens, E., Barani, K., and Karp, E. (1964), *Arch. Biochem. Biophys. 106,* 280-293.

Birchmeier, W., Wilson, K. J., and Christen, P. (1973), *J. Biol. Chem. 248,* 1751-1759.

Biro, E. N. A., Coelho, R., Ehrlich, E., Gullain, F., and Dvonc, C. (1973), *Eur. J. Biochem. 40,* 527-531.

Collins, J. H. (1976), *Nature (London) 259,* 699-700.

Cummins, P., and Perry, S. V. (1973), *Biochem. J. 133,* 765-777.

Dreizen, P., Gershman, L. C., Trotta, P. P., and Stracher, A. (1967), *J. Gen. Physiol. 50,* 85-113.

Duke, J., Takashi, R., Ue, K., and Morales, M. F. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 302-306.

Dunker, A. K., and Rueckert, R. R. (1969), *J. Biol. Chem. 244,* 5074-5080.

Fiske, C. H., and SubbaRow, Y. (1925), *J. Biol. Chem. 66,* 375-400.

Frank, G., and Weeds, A. G. (1974), *Eur. J. Biochem. 44,* 317-334.

Gray, R. H., and Steffensen, D. M. (1968), *Anal. Biochem. 24,* 44-53.

Gutfreund, H., and McMurray, C. H. (1970), *Biochem. Soc. Symp. 31,* 39-47.

Huszar, G., and Elzinga, M. (1971), *Biochemistry 10,* 229-236.

Huxley, A. F., and Simmons, R. M. (1971), *Nature (London) 233,* 533-538.

Iwanaga, S., Wallen, P., Gröndahl, N. J., Henschen, A., and Blombäck, B. (1969), *Eur. J. Biochem. 8,* 189-199.

Joernvall, H. (1970), *Eur. J. Biochem. 14,* 521-534.

Jones, J. M., and Perry, S. V. (1966), *Biochem. J. 100,* 120-130.

Kendrick-Jones, J. (1974), *Nature (London) 249,* 631-634.

Kominz, D. R., Hough, A., Symonds, P., and Laki, K., (1954), *Arch. Biochem. Biophys. 50,* 148-159.

Kulbe, K. D. (1974), *Anal. Biochem. 59,* 564-573.

Lowey, S., and Cohen, C. (1962), *J. Mol. Biol. 4,* 293-308.

Lowey, S., Slayter, H. S., Weeds, A. G., and Baker, H. (1969), *J. Mol. Biol. 42,* 1-29.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265-275.

Lymn, R. W., and Taylor, E. W. (1970), *Biochemistry 9,* 2975-2983.

Malik, M. N. and Martonosi, A. (1972), *Arch. Biochem. Biophys. 152,* 243-257.

Mandelkow, E. M., and Mandelkow, E. (1973), *FEBS Lett. 33,* 161-166.

Mannherz, H. G., Barrington-Leigh, J., Holmes K. C., and Rosenbaum, G. (1973), *Nature (London) 241,* 226-229.

Margossian, S. S., Lowey, S., and Barshop, B. (1975), *Nature (London) 258,* 163-166.

Mendelson, R. A., Morales, M. F., and Botts, J. (1973), *Biochemistry 12,* 2250-2255.

Mendez, E., and Lai, C. Y. (1975), *Anal. Biochem. 68,* 47-53.

Morita, F. (1967), *J. Biol. Chem. 242,* 4501-4506.

Mueller, H. (1964), *J. Biol. Chem. 239,* 797-804.

Mueller, H. (1965), *J. Biol. Chem. 240,* 3816-3828.

Murphy, A. J. (1974), *Arch. Biochem. Biophys. 163,* 290-296.

Offer, G. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 87-93.

Pfister, M., Schaub, M. C., Watterson, J. G., Knecht, M., and Waser, P. G. (1975), *Biochim. Biophys. Acta 410,* 193-

209.
Pope, B. J., Wagner, P. D., and Weeds, A. G. (1977), *J. Mol. Biol. 109,* 470-473.
Reisler, E., Burke, M., and Harrington, W. F. (1974), *Biochemistry 13,* 2014-2022.
Sarkar, S. (1972), *Cold Spring Harbor Symp. Quant. Biol. 37,* 14-17.
Schaub, M. C., and Perry, S. V. (1971), *Biochem. J. 123,* 367-377.
Schaub, M. C., Watterson, J. G., and Waser, P. G. (1975), *Hoppe-Seyler's Z. Physiol. Chem. 356,* 325-339.
Seidel, J. C., and Gergely, J. (1971), *Biochem. Biophys. Res. Commun. 44,* 826-830.
Sekine, T., and Yamaguchi, M. (1963), *J. Biochem. (Tokyo) 54,* 196-198.
Sekine, T., Barnett, L. M., and Kielley, W. W. (1962), *J. Biol. Chem. 237,* 2769-2772.
Sobieszek, A., and Bremel, R. D. (1975), *Eur. J. Biochem. 55,* 49-60.
Starr, R., and Offer, G. (1973), *J. Mol. Biol. 81,* 17-31.
Strauch, L. (1965), *Z. Klin. Chem. Klin. Biochem. 3,* 165-167.
Taylor, E. W., Lymn, R. W., and Moll, G. (1970), *Biochemistry 9,* 2984-2991.
Thomas, D. D., Seidel, J. C., Hyde, J. S., and Gergely, J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1729-1733.
Trentham, D. R., Bardsley, R. G., Eccleston, J. F., and Weeds, A. G. (1972), *Biochem. J. 126,* 635-644.
Wagner, P. D., and Yount, R. G. (1975a), *Biochemistry 14,* 1900-1907.
Wagner, P. D., and Yount, R. G. (1975b), *Biochemistry 14,* 5156-5162.
Watterson, J. G., and Schaub, M. C. (1973), *Hoppe-Seyler's Z. Physiol. Chem. 354,* 1619-1625.
Watterson, J. G., Schaub, M. C., Locher, R., Di Pierri, S., and Kutzer, M. (1975), *Eur. J. Biochem. 56,* 79-90.
Weber, A., and Murray, J. M. (1973), *Physiol. Rev. 53,* 612-673.
Weeds, A. G., and Lowey, S. (1971), *J. Mol. Biol. 61,* 701-725.
Weeds, A. G., and Pope, B. (1977), *J. Mol. Biol. 111,* 129-157.
Weeds, A. G., and Taylor, R. S. (1975), *Nature (London) 257,* 54-56.
Weeds, A. G., Hall, R., and Spurway, N. C. (1975), *FEBS Lett. 49,* 320-324.
Werber, M. M., Szent-Györgyi, A. G., and Fasman, G. D. (1972), *Biochemistry 11,* 2872-2883.
Yount, R. G., Ojala, D., and Babcock, D. (1971), *Biochemistry 10,* 2490-2496.

Modulator Protein as a Component of the Myosin Light Chain Kinase from Chicken Gizzard†

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ABSTRACT: The Ca^{2+}-dependent regulation of smooth muscle actomyosin involves a myosin light chain kinase (ATP:myosin light chain phosphotransferase). It has been shown (Dabrowska, R., Aromatorio, D., Sherry, J. M. F., and Hartshorne, D. J. 1977, *Biochem. Biophys. Res. Commun. 78,* 1263) that the kinase is composed of two proteins of approximate molecular weights 105 000 and 17 000. In this communication it is demonstrated that the 17 000 component is the modulator protein. This conclusion is based on: (1) the identical behavior of the 17 000 kinase component and modulator protein in assays of actomyosin Mg^{2+}-ATPase activity, phosphorylation of myosin, and phosphodiesterase activity, and, (2) the similarity of the 17 000 kinase component and the modulator protein with respect to amino acid composition, absorption spectrum, and electrophoresis in urea-polyacrylamide gels. It is shown also that the modulator protein from smooth muscle and troponin C are distinct proteins.

The regulation by Ca^{2+} of the actin-myosin interaction in smooth muscle is thought to be due to the concerted action of a protein kinase and a phosphatase (Gorecka et al., 1976; Sobieszek, 1977; Chacko et al., 1977). In the presence of Ca^{2+} the kinase phosphorylates the 20 000-dalton light chain of myosin (1 mol of phosphate/light chain) and thereby allows the activation by actin of the Mg^{2+}-ATPase activity. As long as Ca^{2+} is present cyclic actin-myosin interactions proceed with the concurrent hydrolysis of ATP and development of tension or shortening. In the absence of Ca^{2+}, i.e., when the muscle relaxes, the protein kinase is not active and the phosphatase removes the phosphate groups from the myosin molecule. This prevents the actin-activation of ATPase activity and the contractile apparatus is turned "off". Thus the regulatory proteins of smooth muscle are the kinase and the phosphatase and are quite different from the troponin-tropomyosin system in skeletal muscle. It should be pointed out that, although the phosphorylation theory has received wide support, it is not unanimously accepted. Head et al. (1977) have suggested that a troponin-like mechanism is involved in the regulation of smooth muscle activity, and Mikawa et al. (1977) have isolated an activator which does not phosphorylate myosin.

In an attempt to establish the correlation between the activation of ATPase activity and the phosphorylation of myosin we recently isolated the myosin light chain kinase. Our rea-

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